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FETAL RAT ADRENAL STEROIDOGENESIS

STEVEN M. KALAVSKY

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FETAL RAT ADRENAL STEROIDOGENESIS

by

Steven M. Kulavsky

A Thesis

Submitted in Partial Fulfillment

of the Requirements for the Degree

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INTRODUCTION

The purpose of this experiment was to study the development of adrenal biosynthesis of corticosteroids in the rat fetus.

Rat vs Human Adrenal Cortex

The rat adrenal differs from the human adrenal in several ways. The primary secretory product of the adult rat adrenal cortex is corticosterone but in the human adrenal it is cortisol (41). This biochemical difference is not reflected by any histological differences between the adult rat and human adrenals--both are composed of three clearly defined zones. But there are histological differences between the two species during and immediately after the fetal stage of development. The fetal rat adrenal cortex does not develop a "fetal zone" which is clearly found in the human fetus (20, 40). In the human the "fetal zone" undergoes degeneration immediately after birth and eventually disappears, causing the adrenal to shrink. In the rat adrenal the entire cortex--not just a single zone--shrinks after birth because mitotic activity is counter-balanced by visible cellular degeneration in all zones (45). Plate I contains a summary of the histological development of the fetal rat's adrenal cortex. The fetal rat is born during its 22nd day of gestation and at birth, in spite of a rapid terminal development, it is

slightly more immature than the human.

Fortunately the rat provides ready access to large numbers of fetuses with a timed gestation. Previous investigations have already been published indicating that the fetal glands were active in steroidogenesis.

Rat Fetal Pituitary-adrenal Axis

The first evidence of fetal corticosteroid production was indirect and was based on the demonstration of a "fetal pituitary-adrenal axis" similar to that found in the adult rat. The secretory products of the adrenal act as "feedback controls" causing the pituitary to alter the functional state of the adrenals. Decapitation of fetal rats in the last third of gestation produced: 1) a decrease in adrenal volume (47, 48, 49, 21, 22, 15, 26, 38) and 2) a decrease in adrenal weight (10, 11). It was demonstrated that some of these effects were probably not merely a generalized reaction caused by the removal of the fetus' entire head, but rather caused by the removal of the pituitary because it was observed that ACTH could reverse these effects (25, 27). The fact that corticosteroids were being secreted and acting as feedback controls was further suggested by the observation that excess corticosteroids given to intact fetuses produced adrenal atrophy similar to that produced by decapitation (23). Furthermore unilateral adrenalectomy of 19 day old rat fetuses caused enlargement of the contralateral adrenal. This effect could

be prevented by the administration of cortisone (36). These experiments are discussed in detail in a recent review article (25). Finally it was shown by bioassay measurements that stress (sham adrenalectomy) and hydrocortisone administration at 19 days of gestation could reduce the adrenocorticotropic activity of the pituitaries of 22 day old rat fetuses (43). The difficulty with this indirect method of determining the fetal adrenal status is that it requires a fetal pituitary with functional capabilities similar to those of the adult. The evidence that this is the fact is that 21 day old fetal pituitary tissue can, like ACTH, induce corticosterone production in vitro in adrenal tissue (12). There is no direct evidence that ACTH is produced before 21 days but another parameter indicates that the pituitary is active before this date. Growth hormone, determined by bioassay, is present in the 19 day old fetal pituitary (12). All these experiments suggest that, during the last few days of gestation, the fetal rat possesses a pituitary-adrenal servomechanism similar to the adult.

Rat Corticosteroid Biosynthesis

The fetal adrenal cortex does possess the various enzymes required by the biochemical steps involved in corticosteroid synthesis (summarized in Plate II). 3 B-ol steroid dehydrogenase activity was observed in 12 day old fetal rat adrenal glands (8) and progest-

erone 17 hydroxylase is present at 17 days of gestation (42). The presence of other enzymes (21, 18 and 11B hydroxylases) is expressed by the in vitro production of corticosterone at 18 days of gestation and of 18 oxycorticosterone at 20 days (42). Corticosterone (identified by chromatographic mobility, UV absorption and chromatographic mobility with radioactive corticosterone) was produced in vitro in two samples of pooled fetal rat adrenal tissue. The first sample contained tissue from 13.5-15.5 days and the second contained tissue from 19-22 days days (40).

In the only attempt at specific quantification it was shown that pooled fetal adrenal tissue from 12.5-16 day old fetuses could produce total fluorogens at levels equal to those of adult samples (40). It is apparent that fetal rat adrenals do produce steroids but a clear picture of the quantitative development of biochemical function has not been produced.

Experimental Design

Ideally an accurate knowledge of fetal steroidogenesis requires in vivo measurement, at each stage of development, of the exact amounts of each corticosteroid produced by the fetal adrenal cortex. In vivo measurements are preferable because in vitro measurements, although they are valuable for demonstration of production capacities, do not measure the gland's actual production in the intact functioning organism. An example of the disparity which can occur between in vitro and in vivo

tests is the following demonstration in adult rat adrenals. In vivo measurement of venous adrenal effluent blood 30 minutes after ACTH stimulation demonstrated a 33% increase of corticosterone but measurement of in vitro production under the same conditions revealed a 59% increase after the same time period (44).

Furthermore each separate developmental stage should be measured. Previous studies grouped adrenal tissue of different days of gestation. This "vertical" pooling obscures any day to day variations which may be present. On the other hand, grouping adrenal tissue of the same day of gestation but from different animals ("horizontal" pooling) obscures the variation among animals. Both types of pooling were necessary in previous studies because of the small amounts of steroid produced and the relative insensitivity of the acid fluorescence measurement method. This relative insensitivity is based on two factors: 1) after the extraction and purification procedures it is necessary to recover at least 50mμg of steroid (which is a large quantity when dealing with fetal tissue) and 2) several non-steroidal fluorogens have been found in organic material. At least one of these has a fluorescence development time similar to corticosteroids (13).

Theoretically it would be necessary to measure separately each steroid produced. Because of the relatively large quantity of steroid required by the acid fluorescence method it was necessary to combine all the

steroids produced at a given time period. Therefore it was impossible to elucidate biosynthetic pathways because it was impossible to measure each steroid and its precursor separately.

This study was undertaken because the primary difficulty--the lack of a method sensitive enough to detect the amounts of steroids being produced in the fetus--was lessened by the development of a method using the steroid binding properties of corticosteroid binding globulin (CBG) in the measurement of various steroids in body fluids (35). The method is rapid--requiring about 3 hours--and capable of measuring small amounts of steroids--100-500x more sensitive than the acid fluorescence method (34). Furthermore the method is highly specific (with only slight interference from prednisone and 6-methylprednisone and even less from various androgens and estrogens) and free from non-steroidal interference (such as thyroxine, vitamins, and numerous miscellaneous compounds) (34). With thin-layer chromatography, the separate measurement of corticosterone, desoxycorticosterone, cortisol and desoxycortisol is possible. Minor modifications, which are described in the MATERIALS AND METHODS section, had to be made for the purpose of this study.

As in previous experiments, the size of the fetus was the major limiting feature in experimental design. It was impossible to study the difference between adrenal arterial and venous blood concentrations of the corti-

costeroids. It was necessary to measure the concentration of corticosteroids in carefully dissected, "horizontally" pooled fetal adrenal tissue because of the small size of fetal adrenals. It is not clear what correlation exists between the concentration of steroid in adrenal tissue and the amount and rate of production of steroid synthesized in the tissue. In the adult pig and dog adrenal the amounts of 11 B-OH-progesterone and androsterone present in glands were equal to the amounts secreted in 1-2 minutes (17). When ACTH stimulation was blocked with ethyl tryptamine both the concentration in the adrenal tissue and the concentration in venous effluent blood decreased in a parallel fashion (17). If the dynamics are similar in the fetal rat adrenal, the concentration of corticosteroids in the fetal tissue should give an accurate indication of in vivo production.

The most immature tissue examined in this study is from fetuses of 18 days of gestation because at this stage the adrenal tissue is discrete and easily dissected free with little extraneous tissue. It was decided to examine corticosterone, the prime secretory product of the adult rat and also cortisol to determine which steroid was predominant in the fetus. To further define the relative prevelance of the biosynthetic pathways of these two compounds, it was decided also to examine their biochemical precursors, desoxycorticosterone and desoxycortisol.

I. MATERIALS*

Radioactive Steroids

Corticosterone-1,2-T**, molecular weight 346.5, of specific activity 39.1 curies/mM was purified twice by thin layer chromatography on silica gel G in 1) chloroform:acetone (20:80)--greater than 98% radiochemical purity and 2) chloroform:ethanol (70:30)--greater than 98% radiochemical purity. Further purification was accomplished by paper chromatography in light petroleum:toluene:methanol:water (50:50:70:30)--greater than 98% radiochemical purity. The tritiated corticosterone was stored in penicillin type bottles in 100% ethanol at +2°C. When ready for use, a solution of 20μc/ml of benzene:methanol (9:1) was made up and stored at -10°C.

Steroids

1) 4-pregnene-17a-21-diol-3,20-dione (desoxycortisol) lot # 2645***

2) 4-pregnen-11B,17a,21-triol-3,20-dione (cortisol) lot #T1812, specific rotation +155° in dioxane***

3) 4-pregnen-21-ol-3,20-dione (desoxycorticosterone) lot #T2351, specific rotation +185° in dioxane***

*All chemicals were of "analytical grade" unless stated otherwise

**Supplied by Nuclear-Chicago, Des plaines, Ill.

***Supplied by Ikapharm, Ramat-Gan, Israel

****Supplied by Mann Research Laboratories, N.Y., N.Y.

4) 4-pregnen-11B,21-diol-3,20-dione (corticosterone) lot #S3774. specific rotation +195.3° in dioxane* When ready for use, a solution of 1μg/ml of ethanol was prepared and stored at 6°C.

Corticosteroid binding globulin (CBG)

Blood samples anti-coagulated with heparin were drawn from normal human females in their last trimester of pregnancy. The blood was centrifuged at 0°C at 2000 rpm for 15 minutes. 0.5 ml aliquots of plasma--to be used as the source of CBG-were drawn off and stored at -10°C.

Phosphate buffer solution (used in CBG assay)

27.8gm of NaH_2PO_4 was made up to 1 liter with distilled water to make solution "A." 53.6gm of Na_2HPO_4 was made up to 1 liter with distilled water to make solution "B." Both solutions were stored at 6°C. Immediately before use they were mixed together in the ratio 87.7:12.3::A:B to give a solution with a pH of 6.

Activated florisil (used in CBG assay)

Activation of the florisil (a magnesia-silica gel catalyst, 60-100 mesh**)was carried out by heating at 600°C for 30 minutes. After cooling to room temperature, the adsorbant was stored in a dessicator until use.

*Supplied by Mann Research Laboratories,N.Y.,N.Y.
**Supplied by Fischer Scientific Co.,New Jersey

Modified Bray's solution

Because the water soluble CBG would be insoluble in the original toluene scintillation fluid developed by Bray, it was necessary to use the following modified solution (39):

> 100 gm of naphthalene, 7.0 gm PPO and 0.3 gm POPOP were dissolved in 1 liter of dioxane and stored in a dark room.

Tissue

Adult adrenals

Adult male and non-gravid female Sprague-Dawley rats (200-300gm) were sacrificed using an overdose of chloroform. The adrenals were removed, trimmed, weighed and immediately stored in 2 ml of acetone at -10°C.

Fetal adrenals

Gravid Sprague-Dawley rats were obtained fromm Camm Research Co., New Jersey. The first day of gestation was determined as that day during which sperm plugs were identified in the vagina of a female exposed to a male the preceding night. The animals were kept two in a cage and fed with water *ad lib* and laboratory rat chow. They were sacrificed with an overdose of chloroform on gestation days 18, 19, 20, 21 and 22. The 22 day old "fetuses" were actually newborn. The gestational age was confirmed by several quantitative and qualitative parameters summarized in Plate III. These parameters were in agreement with those previously published (50). Plate III also contains the number of fetuses studied at each age. Some of the gravid animals were pseudo-pregnant and others showed

evidence of intra-uterine infection. If fetuses were foul-smelling, undergoing lysis and/or less than 10 mm in crown-rump length, they were not included in this study. Unfortunately only 1 gravid rat of 20 days of gestation was in good condition. Because of this, 1) insufficient fetal adrenal tissue was obtained for this day and 2) all the tissue was from siblings. Therefore the values for fetal adrenal tissue of 20 days gestation are not included in this study.

The gravid uterus of each animal was removed through a ventral incision immediately after death. Each uterus was opened and each fetus with its placenta and fetal membranes was dissected free under direct visualization. The umbilical vessels were cut at the fetal body wall and the placentae, with umbilical vessels and membranes attached, were placed in a 25 ml beaker immersed in an ice water bath and covered with a saline soaked sponge for 5-15 minutes until they could be weighed. To remove the fetal adrenals a ventral horizontal incision was made at the bottom of the rib cage and the adrenals were identified under 10x magnification, removed and trimmed of extra-adrenal tissue. The adrenals of the same litter were weighed immediately and placed in a test tube containing 1 ml of acetone and immersed in an icewater bath until all adrenal tissue from the same day of gestation was pooled in the tube.

Homogenization

All tissue was homogenized in a Waring Blender.

1. The fetal bodies and placentae were placed in the homogenization chamber, but the fetal and adult adrenals were placed in the micro-attachment.
2. 1 ml of acetone/mg of tissue or at least 2 ml of acetone was added and the mixture was homogenized for 2 minutes at 30 second bursts.

When examined under a microscope at 1000x no intact cells were observed in fetal body, placenta or adult adrenal tissue homogenized under these conditions.

3. All samples were stored at -10°C.

Deproteinization and extraction

The tissue samples to be assayed contained an unknown amount of protein, at least some of which presumably possessed steroid binding sites. If this protein were not removed from the samples an unknown increment of steroid binding sites would be added to each unknown and it would be impossible to guarantee that an equal amount of binding sites would be added to each sample. To prevent this, protein was removed from the solution by precipitation

1. Tissue samples were brought to room temperature.
2. 2 ml of acetone were added and the mixture was shaken by hand for 5 minutes.
3. The mixture was then passed through a #2 Whatman filter

paper to remove the precipitated protein.

4. The storage vessels and the filter paper were washed with two 5 ml aliquots of acetone and the wash aliquots were added to the filtrate.
5. The filtrate was brought to dryness at 42°C in a constant temperature water bath.

Isolation of steroids

Because each of the steroids to be assayed competed for the same bimding site it was necessary to measure each one in a separate solution. To prepare these separate solutions, corticosterone, desoxycorticosterone, cortisol and desoxycortisol were separated by thin layer chromatography. ChromAR Sheet* was used with the following developing solutions:

#1 chloroform:methanol:water (97:3:.5)
#2 chloroform
#3 chloroform:ethyl acetate:water (50:50:1)

The location of control steroids was achieved by observation under short wave length UV light. The R_f's for each steroidare summarized in Plate IV.

1. The dried samples were reconstituted with 0.2 ml ethyl acetate and spotted on what was arbitrarily defined as the left lower corner of an 8x8 inch sheet of ChromAR Sheet (Plate V, Step 1).
2. Transfer was completed by two additional washes with 0.2 ml aliquots of ethyl acetate.
3. 5µg samples of standard corticosterone, desoxycorticosterone, cortisol and desoxycortisol were spotted on

*Supplied by Mallinkrodt

the lower right hand corner--all samples on the same spot (Plate V, Step 2).

4. The sheet was then developed in the first dimension using solution #1 (Plate V, Step 3) and then dried at room temperature.
5. To prepare the sheet for development in a second dimension a 5µg sample of standard desoxycorticosterone was then spotted at the original upper left hand corner of the sheet on a line running through the original assay spot and parallel to the edge of the sheet (Plate V, Step 4).
6. 5µg samples of cortisol, corticosterone and desoxycortisol were spotted in the original lower left hand corner of the sheet about 2 cm below the point where the original samples were spotted (Plate V, Step 5).
7. The sheet was then observed under short UV light and on the basis of fluoresdence and R_f values the desoxycorticosterone controls and unknowns were cut from the cortisol, corticosterone and cortisol controls and unknowns (Plate V, Step 6).
8. The portion of the sheet containing desoxycorticosterone was then developed in a second dimension, perpendicular to the first dimension, in solution #2 (Plate V, Step 7a).
9. The portion of the sheet containing cortisol, corticosterone and desoxycortisol was also developed in a second dimension perpendicular to the first in solution #3 (Plate V, Step 7b).
10. After drying, both portions were observed under short

wave-length UV light and the locations of the sample steroids was determined by coordinating the locations of the control steroids. Once identified, each steroid to be assayed was removed from the sheet by cutting a 2.5x2.5 cm square from around the point where that steroid was located.

Elution

1. Each square was hooked onto a 16G needle which was bent to an angle of 20°.
2. The needle was placed on a syringe which was then filled with 5 ml ethyl acetate and the solvent was allowed to drip out of the needle and through the squares by gravity.
3. The eluate was collected in a test tube and then dried at 42°C.
4. The samples were then reconstituted with 1 ml of ethanol.

Corticosteroid binding technique

The first step in this method is the preparation of a standard assay solution, containing CBG saturated with a tritiated steroid. Since CBG is elevated during pregnancy, the most convenient source of CBG is human plasma during the last trimester of pregnancy. When preparing the solution, if too much plasma is added, the small amounts of unknown steroid displace such a small percentage of tritiated steroid from the CBG that the change in radioactivity is undetable. Thus

decreasing the concentration of plasma increases the sensitivity of the method. But as one adds less plasma, the amount of radioactivity that can be bound by the CBG decreases, approaching background, and the slope of the resultant standard curve approaches zero. The problem becomes one of determining how little CBG one should add. It was found that a 0.25 vol % solution produced a slope that was too flat (34). A 0.5 vol % solution was tried in this investigation and found satisfactory. The solution is made up in a buffer solvent which provides a pH at which CBG is most stable.

To complete the assay solution, the steroid binding sites of CBG must be filled with a tritiated steroid. No matter what particular steroid is to be measured, all the corticosteroids bind at the same site and one tritiated steroid -- in this case corticosterone -- can be used to fill this site. The exact amount of tritiated steroid is not critical; 4μc% was selected because it produced convenient counting times. In practice, the tritiated steroid must be added to, and diluted by, the buffer before the CBG so that its organic solvent is diluted enough to prevent the precipitation of the water soluble CBG.

Once the standard assay solution is prepared, an equal amount is added to each unknown steroid. To optimize conditions for equilibration, this solution must be incubated for 30 minutes at 42°C allowing complete dissolution of all constituents, the solution is then brought to 6°C for at least 15 minutes bringing

the solution to an optimum temperature at which the equilibration of tritiated and unknown steroids can occur.

After equilibration has occured it is necessary to remove all steroids not bound to the CBG with an adsorbant (florisil). The adsorption characteristics of varying amounts of florisil is demonstrated in Plate VI. It compares the effect of florisil in two different solutions--one containing CBG and the other without CBG. It is apparent that up to 80 mg of florisil can adsorb, at most, 70% of the tritiated steroid in a solution which does not contain CBG, but adsorbs much less in a solution which contains CBG. The difference between the two curves is greatest at 40 mg of florisil, at which point about 30% of the steroid appears to be bound to the CBG. Theoretically, amounts of florisil less than 40 mg do not remove a maximum amount of unbound steroid from the solution and amounts greater than 40 mg "pull steroid off the binding sites" by removing so much unbound steroid that the equilibration reaction is shifted in the direction of unbound steroid. For these reasons 40 mg of florisil was chosen as the optimum amount of adsorbant. After the adsorbant is added the solution is vigorously shaken for 2 minutes allowing maximum exposure of the constituents.

As mentioned in the introduction, the amount of tritiated steroid remaining on the CBG is inversely related to the amount of unknown steroid. Thus when one prepares a standard curve, relating radioactivity to known amounts of each steroid, plotting the number

of counts / unit of time would produce a curve with a negative slope. This investigation utilizes a plot of unit of time / number of counts which produces a positive slope. Because each steroid has a different affinity for CBG, it is necessary to prepare a standard curve for each steroid to be assayed.

1. Material for a standard curve was prepared in duplicate by adding known amounts of corticosterone, cortisol, desoxycortisol, and desoxycorticosterone, in quantities from 0 to 1.5 mµg, from stock solutions made to 100mµg/ml and the tubes were then brought to dryness at 42°C.
2. Known volumes of the steroids to be assayed (dissolved in ethanol as described above) were then added to test tubes in duplicates and were brought to dryness at 42°C.

From this point, tubes from 1. and 2. were placed in a test tube rack and handled in the same manner.

3. 1 ml of a solution containing 4µc% of tritiated corticosterone and 0.5 vol % human late pregnancy plasma dissolved in the phosphate buffer was then added to each tube.
4. The tubes were incubated at 42°C for 30 minutes.
5. The tubes were then transferred to a constant temperature bath at 6°C and allowed to equilibrate for at least 15 minutes.
6. 40 mg of activated florisil was added to each tube with a volumetric spatula designed to deliver 40 mg of florisil.

7. The tubes were then shaken at a rate of 120 cycles/ minute for two minutes in an automatic Arthur H. Thomas Co. shaker.
8. The tubes were then allowed to stand at $6^{o}C$ for 30 minutes to allow the florisil to settle.
9. 0.5 ml from each tube was pipetted into a plastic counting vial.
10. 5 ml of modified Bray's solution were added to each vial
11. The samples were counted in a Packard Tri-Carb liquid scintillation counter for 10^{4} counts. Results were in minutes / 10^{4} counts.

Statistical methods

Methods described in statistics textbooks were used to calculate the standard curves by the least squares method, prove their linearity, calcuate mean concentrations and 95% confidence limits (6), and test the difference between means (51).

RESULTS

Standard curves

The standard curves and unknown values were derived from four observations at each concentration. The standard curves determined by the corticosteroid binding globulin technique are not linear throughout their entire length. The slope approaches zero at both extremes of high and low concentrations of steroids (see Plate VII) but at the middle portion of the standard curves for cortisol, corticosterone, desoxycortisol and desoxycorticosterone a straight line was determined by the method of least squares which derives a straight line such that, at each point observation on the abscissa, the sums of the squares of the deviations of the observed values from the straight line are at a minimum. The straight line is defined by calculating the slope and y-intercept by solving the following two equations:

$$1.\ \Sigma Y = na + b\Sigma X$$
$$2.\ \Sigma XY = a\Sigma X + b\Sigma X^2$$

where a = y-intercept, b = slope, ΣY = the sum of all the observations, n = the number of concentrations measured, ΣX_2= the sum of all the concentrations measured, ΣX^2 = the sum of the squares of all the concentrations measured and ΣXY = the sum of the products of the observations and concentrations measured (51).

The variance (which is the square of the standard deviation) is determined by solving the following equation:

$$\text{variance} = \sum \frac{(\sum Y)^2}{k} - \frac{(\sum \sum Y)^2}{\sum k} - \frac{(\sum k(\bar{X} - X)(\bar{Y} - Y))^2}{\sum k(X - \bar{X})^2}$$

where $\sum Y$ = the sum of the observations at each point, $\sum \sum Y$ = the total sum of observations, k = the number of observations at each point, $\sum k$ = the total sum of the number of observations, $\bar{X}$ = the mean concentration measured, $\bar{Y}$ = the mean of the observations at each point, X = the concentration measured and Y = the observed value (6).

The test for linearity of the standard curves was done by dividing the mean variances of the observed values about the line by the mean variances within groups which provides the Fischer Variance Ratio, F. For all the standard curves the F value was demonstrated to be below the 0.90 point on the standard F distribution so that the hypothesis that the curves were linear was acceptable (6). The ranges of linearity for the standard curve of each steroid, as well as a representative slope and standard deviation, are summarized in Plate VIII. Plate IX contains representative standard curves for cortisol, corticosterone, desoxycortisol and desoxycorticosterone.

Adult Rat Adrenal Glands

Corticosterone is present in largest amounts (769 ± 278mμg extractable steroid/100mg adrenal tissue) and desoxycorticosterone is the next most plentiful steroid (287 ± 207mμg/100mg). Low but similar concentrations were found for cortisol (69.4 ± 2.7mμg/100mg) and desoxycortisol (66.5 ± 35.1mμg/100mg). The ratio of corticosterone/cortisol = 9.8 and the ratio of desoxycorticosterone/desoxycortisol = 4.3. In rat plasma the

ratio of corticosterone/cortisol = 20 (7) and in human plasma the ratio = .085 (5).

To compare these values with other published data it was necessary to determine the percentage of steroid lost during the extraction procedure. Since the published values were corrected by use of an external control it was decided to use the same type of control. This was done by adding a known amount of tritiated corticosterone to the storage vessel of an adult adrenal gland. After carrying the tissue through the entire preparative procedure: extraction, deproteinization, thin layer chromatography and elution, 44% of the tritiated material was recovered. The amount of total corticosterone per 100mg adrenal tissue, using 44% as the recovery percentage, was then determined to be 1.56±0.64µg/100mg. Because the mean weight of the adrenal glands was 20.0mg, this value could also be expressed as 0.312±0.127µg/adrenal gland. This compares well with the value of 0.5µg corticosterone/adult rat adrenal determined by fluorescent spectrophotometry (16), but this study describes neither the strain of rat studied nor the weight of each adrenal gland so it is difficult to compare the values. The concentration determined in the current study (1.56 ±0.64µg/100mg) is also very similar to the value of 3.0±1.0µg/100mg of adrenal tissue which was determined by UV spectrophotometry in adult male Wister rats (2). Unfortunately there are no published determinations of cortisol, desoxycortisol or desoxycorticosterone.

Since the 44% recovery rate is actually an external control and the actual amount of steroid lost during each extraction is not known, all remaining concentrations in this paper will not be corrected for loss during extraction and will be expressed as mμg of extractable steroid/gm of tissue.

Placental Tissue (Values summarized in Plate X)

No desoxycortisol was detected in placental tissue of any age and desoxycorticosterone was undetectable on the 20th and 21st day of gestation. Corticosterone and cortisol were detected at all ages. The average value of corticosterone was 3.7 mμg/gm of tissue and it did not vary significantly from day to day. The average value of desoxycorticosterone was 4.46 mμg/gm on the 18th and 19th day of gestation. The average value of cortisol on the 18th, 19th and 20th days of gestation was 3.06 mμg/gm but on day 21 it underwent a significant ($P = .005$) rise to 25.1 mμg/gm of tissue (see Plate XI, graph 1).

It should be realized that these extremely small concentrations were detectable only because large amounts of placental tissue were available. As compared to each fetal adrenal sample, which contained .02-.08gm of tissue, each placental sample contained 3-4gm. For the same reason, equally small concentrations were detectable in fetal body homogenates.

Fetal Body Homogenates (Values summarized in Plate X)

No desoxycortisol was detected at any age but cortisol, corticosterone and desoxycorticosterone were detected at all ages. The average concentration of desoxycorticosterone was 1.06mμg/gm tissue and it did not vary significantly from day to day. In a similar fashion corticosterone did not vary significantly from day to day and its average value was 2.44mμg/gm tissue. The values of corticosterone and desoxycorticosterone did not vary significantly from those found in the placental tissue except that desoxycorticosterone was not detectable on the 20th and 21st day in the placenta. The values for cortisol are difficult to interpret. The highest concentration, 30.0mμg/gm tissue, was found on the 18th day of gestation. The concentrations on the following days were all significantly lower (P =.025) but they did not drop in a linear manner (see Plate XII, graph 1). It should be remembered that this drop in concentration is in the opposite direction and of different timing than the change of concentration of cortisol in the placenta. Furthermore only at the 18th and 20th days of gestation are there significant differences between the concentration of cortisol in the placenta and fetal body--the concentration of cortisol in the fetal body on the 18th day (30.0mμg/gm tissue) being larger than that in the placenta (2.78mμg/gm tissue, P =.005) but the concentration in the fetal body on the 20th day (11.4mμg/gm tissue) being smaller than that in the

placenta (25.1mug/gm tissue, P =.01).

Fetal Adrenal Glands (Values summarized in Plate X)

Once again no desoxycortisol was detected in this tissue at any age. Furthermore no cortisol or desoxycorticosterone was detected at the 20th, 21st or 22nd days of gestation. Corticosterone was detected at all ages. 1,572mμg of desoxycorticosterone/gm of adrenal tissue was present on the 18th day of gestation. This concentration was approximately 300 times that found in the placenta (P =.0005) and 1000 times that found in the fetal body (P =.0005). Significantly, desoxycorticosterone was then undetectable in the fetal adrenal gland from the 19th day of gestation to birth. On the 18th day of gestation 1,866mμg of corticosterone/gm adrenal tissue was present. This was approximately 600 times greater than the amount present in the fetal body and placenta at this time (P =.0005). Unlike desoxycorticosterone, corticosterone stayed at least at this level for the remainder of gestation. On the 19th day of gestation the concentration of corticosterone underwent a tremendous increase to 7,505mμg/gm adrenal tissue (see Plate XIII, graph 2). Although the change of concentration in the adrenal tissue itself represents a significant change from the previous day (P =.0005) and is actually at a value slightly greater than the average concentration of the adult adrenal, no change in corticosterone concentration is detectable in the fetal body or placenta

(see Plate XII, graph 2 and Plate XI, graph 2). The concentration then drops significantly (P =.0005) by the 20th day of gestation to 1,050mμg/gm tissue and again on the 21st day to 745mμg/gm tissue (a value 1/10th of that found in the adult). The final detectable corticosteroid, cortisol, is present at the 18th day of gestation in the concentration of 2,180mμg/gm tissue, a value 1000 times greater than that in the placenta (P =.0005) and 100 times that in the fetal body (P =.0005). However on the 19th day the amount of cortisol drops significantly (P =.0005) to 287mμg/gm tissue, a value about 1/2 of the adult and then becomes undetectable for the remainder of gestation (see Plate XIII, graph 1). This change in cortisol concentration roughly parallels the change observed in the fetal body but is in exactly the opposite direction of that seen in the placenta (see Plate XI, graph 1 and Plate XII, graph 1).

DISCUSSION

Validity of Technique

The results of this experiment indicate that the corticosteroid binding globulin technique--developed for the assay of steroids in body fluids (34)--is easily adapted to the measurement of steroids in tissue samples. The concentration of corticosterone in adult rat adrenal tissue determined by the CBG method (1.56±0.64µg/100mg) correlates well with previously published values determined by either the colorimetric tetrazole blue method (3.4±µg/100mg (2)) or UV spectrophotometry (3.0±1µg/100mg (2) and 0.5µg/gland (16)). Of course this comparison must be made with the realization that Angelico, et al measured Wister rats, Givner, et al did not specify the strain of rat studied and this study investigated Sprague-Dawley rats. Furthermore the results of the acid fluorescence method can be distorted by non-steroidal fluorescence and the results of the tetrazole blue method, which requires rigorous purification of reagents, can be distorted by any compound which contains an a-keto group, e.g. sugars (13). The fact that the value determined by this investigation was lower than the previous values could therefore be due to species variation, coincidental variations in metabolic states, non-steroidal fluorescence in the UV method or a-ketol

contamination in the tetrazole blue method. However it should be remembered that the CBG method is highly specific for steroids and only a few synthetic steroids are capable of causing slight interference (34). The CBG method also has the advantage of a sensitivity enabling it to measure corticosterone in much smaller samples than other methods--as little as 10 mg of tissue functioning at the level of an adult rat adrenal. It is for these reasons that it lends itself to the study of fetal tissue.

Confirmation of Fetal Steroidogenesis

Turning to the fetal adrenal itself, the fact that steroids are present in concentrations up to 2000 times above those found in the fetal body indicates that either the fetal adrenals store exogenously produced steroids or they are capable of producing endogenous steroids. Taken in conjunction with the experiments mentioned in the introduction, it is apparent that the fetal adrenal gland is capable of secreting cortisol, corticosterone and desoxycorticosterone at, or above, the secretory levels in adult rats.

Phases of Fetal Steroidogenesis

A theory has been proposed suggesting that fetal steroidogenesis occurs in four stages (40). The results of this experiment can be discussed in terms of these four phases.

Phase 1

This phase, from 10.5 to 16 days of gestation, is one in which the fetal adrenal develops toward the adult in terms of structure and function, but is free from pituitary dependence (40). Adrenal activity---measured by total fluorogen secretion in vitro--first reaches adult rat adrenal levels by 12.5 to 16 days of gestation (40). But compounds measured by such non-specific tests are not necessarily the same corticosteroids produced by the adult adrenal. Corticosterone (identified by chromatographic mobility and UV absorption) was first qualitatively identified form 13.5 to 16 days of gestation (40). Although this suggests that the fetal adrenal may be developing functions similar to the adult, there is no evidence that the fetal pituitary is functioning at this time. This phase will not be discussed further here because it was not included in this study.

Phase 2

Roos describes the second phase, from 16 to 18.5 days of gestation, as one in which the pituitary gland causes the differentiated adrenal to grow rapidly but one in which steroid secretion drops (40). Indeed corticosterone becomes undetectable from 16 to 18.5 days of gestation (see Plate XIV, graph 2) (40). Other studies demonstrated that, during the period when corticosterone was undectable, the numbers of mitoses in the gland increase and reach a peak at 18 days of gestation (31). This

spurt in growth makes two observations easily understood: 1) fetal adrenal volume / fetal body weight reaches a maximum at day 19 (31) and 2) fetal adrenal weight / fetal body weight also reaches a maximum around the same time (3) (see Plate XIV, graph 4).

The significance of this peak in mitoses is debatable. Investigations have shown that, in the adult rat adrenal, mitoses are inversely related to steroidogenesis (1, 14, 32, 33) and this seems to be compatible with the disappearance of corticosterone mentioned above. However the amount of total fluorogen recovered in vitro during days 16-18.5 remained at levels equal to that during 12.5-16 days (40) even though corticosterone "disappeared" during this period. The results of this experiment help clarify the situation because, at 18 days of gestation, cortisol and desoxycorticosterone are present at two to three times the amount found in the adult and corticosterone is present in amounts approximately equal to that found in the adult (see Plate V). Thus the "disappearance" of corticosterone may be balanced by a peak in cortisol concentration. A condition similar to this, in which a minor corticosteroid is found in very high levels at one point in fetal development, is also seen in the human. A recent investigation has shown that in 10-15 week old human fetuses (equivalent to 18 day old rat fetuses (50)) the adrenals possess the ability to produce in vitro large amounts of desoxycorticosterone. They do

not possess this ability prior to or after this stage (46).

Thus during the second phase the fetal adrenal seems capable of synthesizing cortisol, corticosterone and desoxycorticosterone in equal amounts (see Plates X and XIII, graphs 1, 2, and 3). The great preponderance of corticosterone in the adult gland has not yet been established and the biosynthetic pathway producing cortisol seems to equal that producing corticosterore. Based on the results of this experiment, Phase 2 may be better characterized as a period of functionally "undifferentiated" tissue which is mitotically active and which, unlike the adult, maintains both cortisol and corticosterone biosynthetic pathways.

Phase 3

Roos describes this phase (from 18.5 days of gestation to birth) as one in which secretion of corticosterone returns (see Plate XIV, graph 2) and in which a new balance between the pituitary and the adrenal is reached (40). The reappearance of corticosterone on day 18.5 was not entirely unexpected. Because of a qualitative increase in lipid content of the fetal adrenal on day 19, two investigators predicted that a "critical period" existed on day 19 which probably indicated an increase in physiological activity (9, 24). Supporting the predicted increase in steroidogenesis on day 19 was the documentation of a fall in concentration

of ascorbic acid on days 19 and 20 (3) (see Plate XIV, graph 3).

The results of this experiment confirm these predictions because, coincidental with the fall in ascorbic acid and rise in adrenocortical lipid, the corticosterone rises 3-fold to a level well above that found in the adult (see Plates X & XIV, graph 1). Furthermore cortisol falls to levels undetectable under these conditions and remains at this low level (see Plates X & XIII, graph 1). This decrease in cortisol concentration may be because of the utilization of common precursors by the tremendous increase in corticosterone concentration. However the fact that it remains low suggests that this may be the stage at which corticosterone becomes the primary secretory product of the adrenal. Because of these changes Phase 3 may therefore be better qualified as involving the establishment of the adult preponderance of the corticosterone pathway over the cortisol pathway.

Phase 4

The fourth phase is the one of much-debated "inertia" starting at birth (40). Evidence suggests that the state of function achieved on day 19 does not remain stable. The change in ascorbic acid in Phase 3 reverses itself on day 20 (see Plate XIV, graph 3) and the concentration increases through day 21 (3) suggesting a decrease in adrenal function. Indeed quite a bit of controversy

exists concerning the state of function of the newborn rat adrenal. Several investigators suggest that the gland is unresponsive to ACTH stimulation (4, 18, 19). One study has demonstrated high levels of corticosterone in the plasma of 21-22 day old rat fetuses which gradually disappear by birth (30). However others suggest that morphological charges do occur on the first postnatal day after ACTH stimulation (37) and another study indicates that 2 day old rats respond to stress by secreting steroids (52). In an attempt to combine these observations it has been suggested that another "critical pericd" exists at birth and handling newborn rats can modify their pituitary-adrenal axis in such a way as to enable their adrenal to respond to stress (28, 29).

This study reveals that, immediately preceding the fourth phase of "inertia", the concentration of corticosterone undergoes a 3-fold decrease at the same time the above-mentioned increase in ascorbic acid occurs (see Plate XIV, graphs 1 & 3). This really adds little to the debate concerning this phase because the decrease merely brings the concentration of corticosterone down to normal adult levels. Indeed this may be the stage during which the pituitary-adrenal axis is stabilized.

A clarification should be made at this point. This experiment is limited to a quantitative documentation of the development of biochemical function and cannot establish causality. Although the results conform to the phasic theory, many features of the

theory--involving the causes of the fluctuations and possible effects--cannot be determined by this study.

Fetal Body Tissues

The steroid concentrations in the homogenized fetal body (see Plates X & XII, graphs 1, 2 and 3) can serve as a "background" for adrenal steroid concentration because they represent the amount of steroid--from whatever source--that is found in tissue which is known not to produce steroids. However the fact that corticosterone and desoxycorticosterone are fixed at low levels and do not fluctuate with adrenal levels (see Plates XII & XIII) is difficult to interpret. Perhaps the various sources of steroids--fetal adrenal, maternal adrenal, placenta--serve to dampen each others fluctuations or perhaps the end organs rapidly convert the steroids to some form which does not bind to CBG. In any case, the variations of cortisol in the fetal body homogenates which roughly parallels the changes in cortisol concentration in the fetal adrenal (see Plates XI & XII) is equally difficult to explain. Perhaps the end organs differ in respect to their handling of different steroids. It is also possible that nonspecific interference with CBG binding from these extracts could have materially lowered values.

Placental Tissues

Placental concentrations of corticosterone and desoxycorticosterone are similar to fetal body concentrations (see Plates XI & XII) but placental cortisol

seems to rise independently at term. This might represent, among other possibilities, either pooling of unmetabolized steroids or the development of the capability to produce cortisol by the placenta. Once again it should be remembered that this investigation can merely document changes and cannot establish causality.

SUMMARY

The corticosteroid binding globulin technique was successfully adapted to the measurement of corticosteroids in fetal tissue samples. Cortisol, corticosterone, desoxycortisol and desoxycorticosterone were measured in adrenal tissue, whole body homogenates and placental tissue of fetal Sprague-Dawley rats on the 18th, 19th, 20th and 21st days of gestation. The results indicate that:

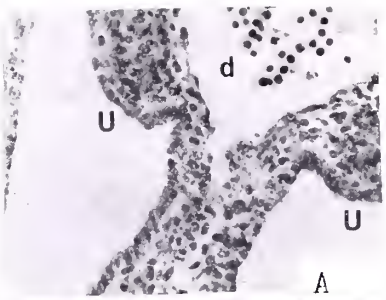
1. Both corticosterone and cortisol biosynthetic pathways seem to be fully developed and function equally by the 18th day of gestation.
2. On day 19 the cortisol biosynthetic pathway becomes depressed.
3. On the same day the corticosterone pathway increases to several times the adult level.
4. By day 21 the corticosterone pathway drops to normal adult levels.

Although this experiment cannot establish causal relationships, the results confirm portions of the phasic theory of fetal steroidogenesis.

Plate I -- Histological development of the fetal rat adrenal cortex

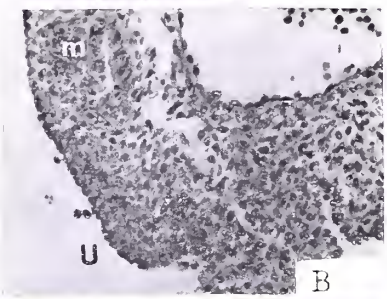
fetal age (days)

11.5 The first histological evidence of differentiated adrenal tissue, a group of rounded, mitotically active, nucleolated cells, is found about 50-100μ behind the cranial end of the urogenital ridge (40).



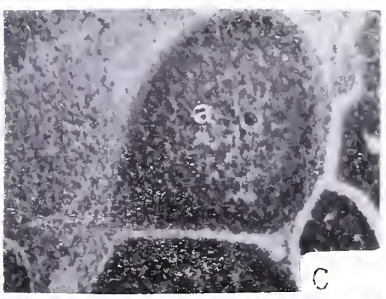
A

13.5 Irregularly discontinous clumps of presumptive adrenocortical cells are seen at the angle of the dorsal mesentary from the level of fusion of the dorsal aortae to the level of the metanephric rudiment and some evidence of capsule formation is evident (40).



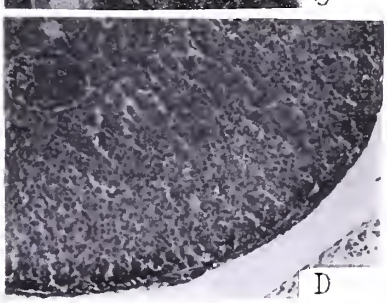
B

15.5 Clumps of tissue start consolidating into loose cords of cortical cells (50) and blood vessels and nerve cells first appear (40).



C

18.5 The gland has an intact capsule and has assumed its characteristic histological arrangement: a basophilic zona glomerulosa, a palisaded zona fasciculata and a zona reticulosa with no evidence of a "fetal zone" similar to that found in primates (20, 40).


D

22.5 After birth the entire cortex shrinks and mitotic activity, which continues, is counterbalanced by visible cellular degeneration in all zones which occurs throughout the first post-natal week (45).

All pictures from Roos '67 (40)
A & B are 80x; C & D are 32x

U = urogenital ridge
d = dorsal aortae
m = metanephros
a = adrenal

Plate II -- Biosynthetic pathway of major adrenal corticosteroids (41)

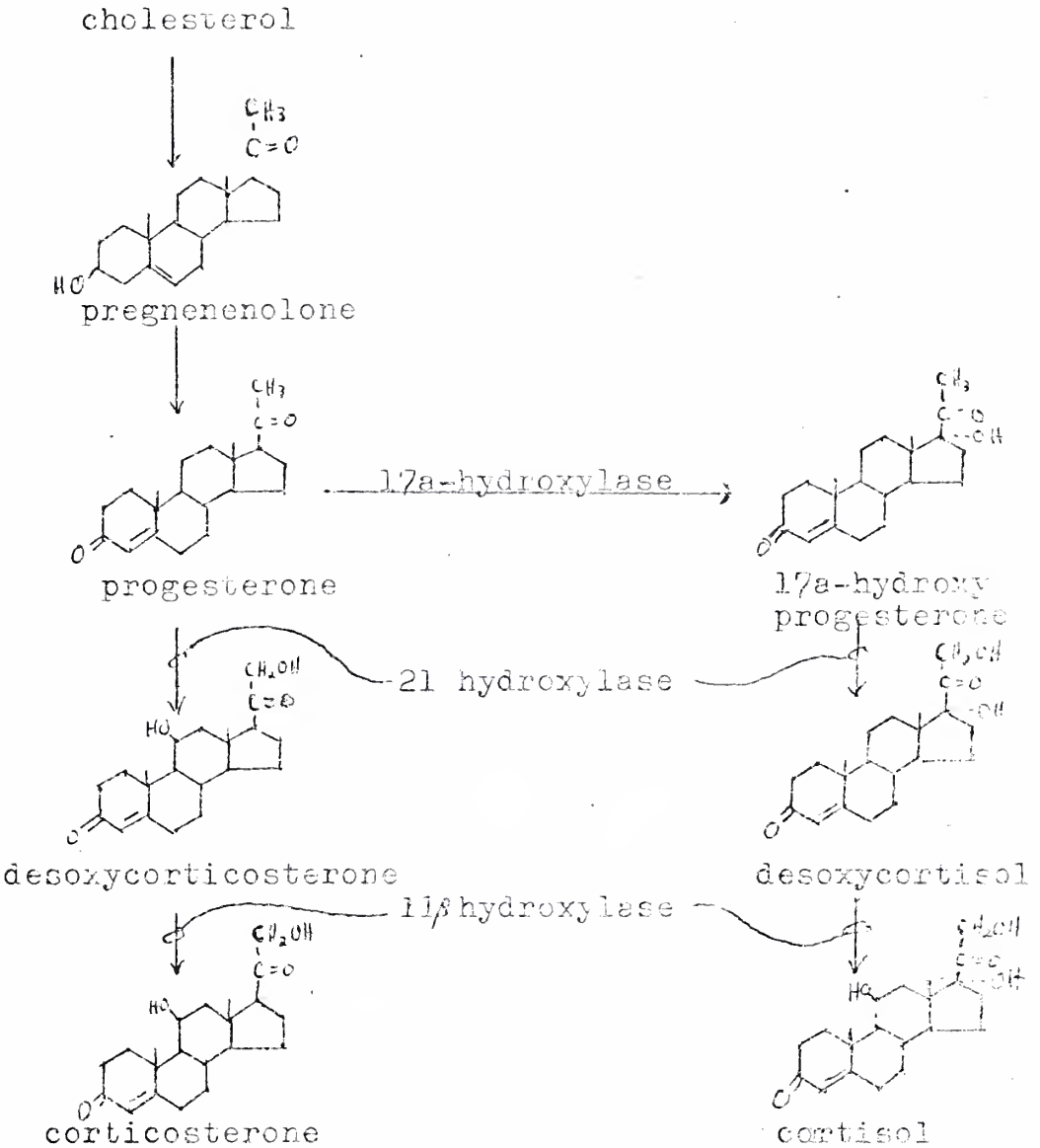


Plate III -- Factors used to confirm gestational age

QUANTITATIVE PARAMETERS OF DEVELOPMENT				
days of gestation	mean C-R length* mm	mean weights mg		
		fetal body	placenta	fetal adrenal
18	17	613	323	.229
19	23	1001	444	.466
20	31	1853	498	.510
21	40	3930	515	1.010
22**	46	5240	---	.708

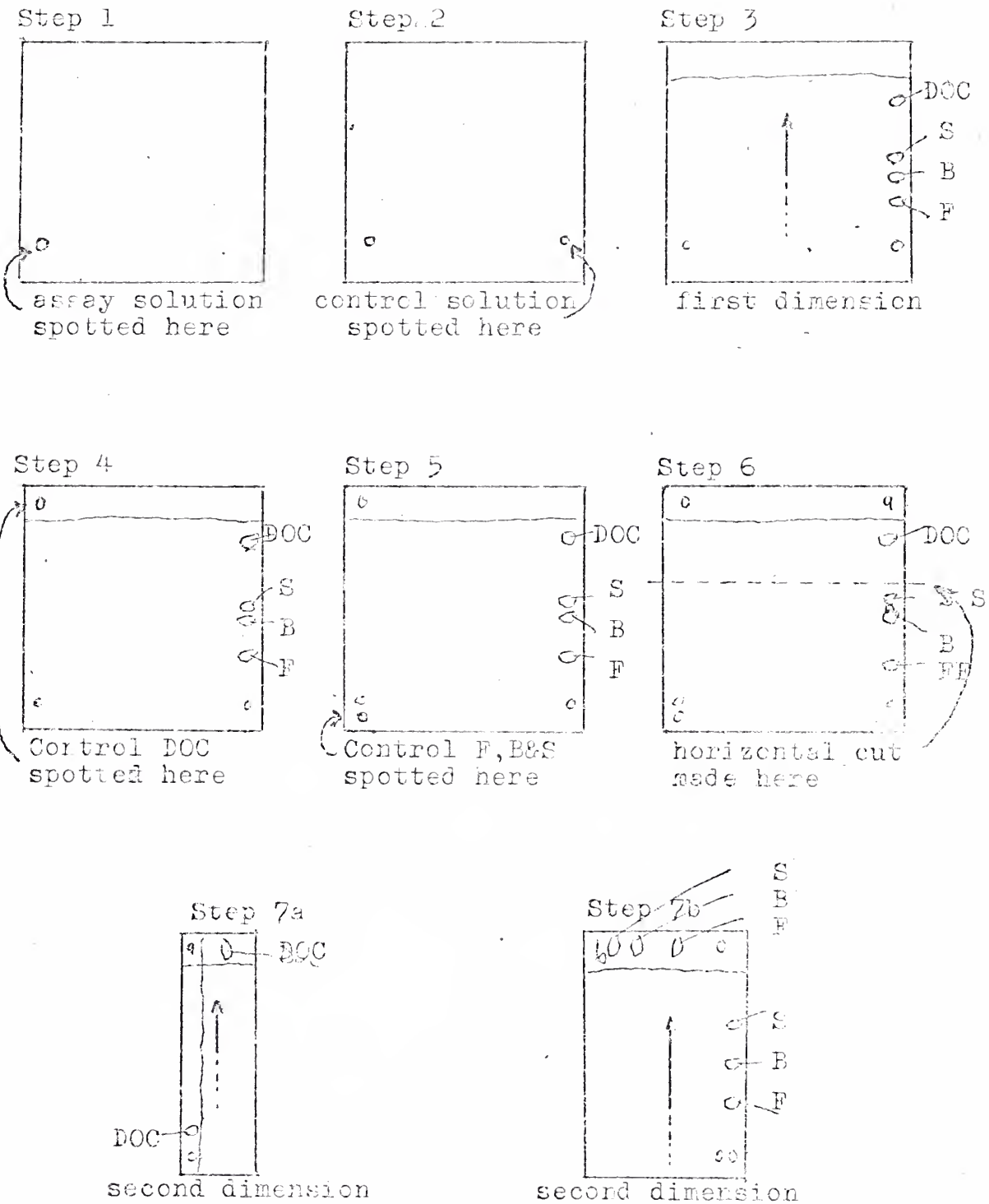
QUALITATIVE PARAMETERS OF DEVELOPMENT				
days of gestation	umbilical hernia	papillae of body hair	formed vibrissae	number of fetuses
18	+	-	-	33
19	-	-	-	53
20	-	+	+	11
21	-	+	+	40
22**	-	+	+	19

*Crown-rump length
**newborn

Plate IV -- R_f's

solvent			deoxy-corticosterone	corticosterone	desoxycortisol	cortisol
#1	chloroform	97	.89-.95	.50-.56	.56-.62	.26-.30
	methanol	3				
	water	.5				
#2	chloroform		.38-.41	.10-.13	.30-.33	.10-.13
#3	chloroform	50	.95-.98	.55-.61	.70-.78	.30-.44
	ethyl acetate	50				
	water	1				

Plate V -- Thin Layer Chromatography



DOC = deoxycorticosterone
S = desoxycorticol
B = corticosterone
F = cortisol

Plate VI -- Adsorption characteristics of florisil

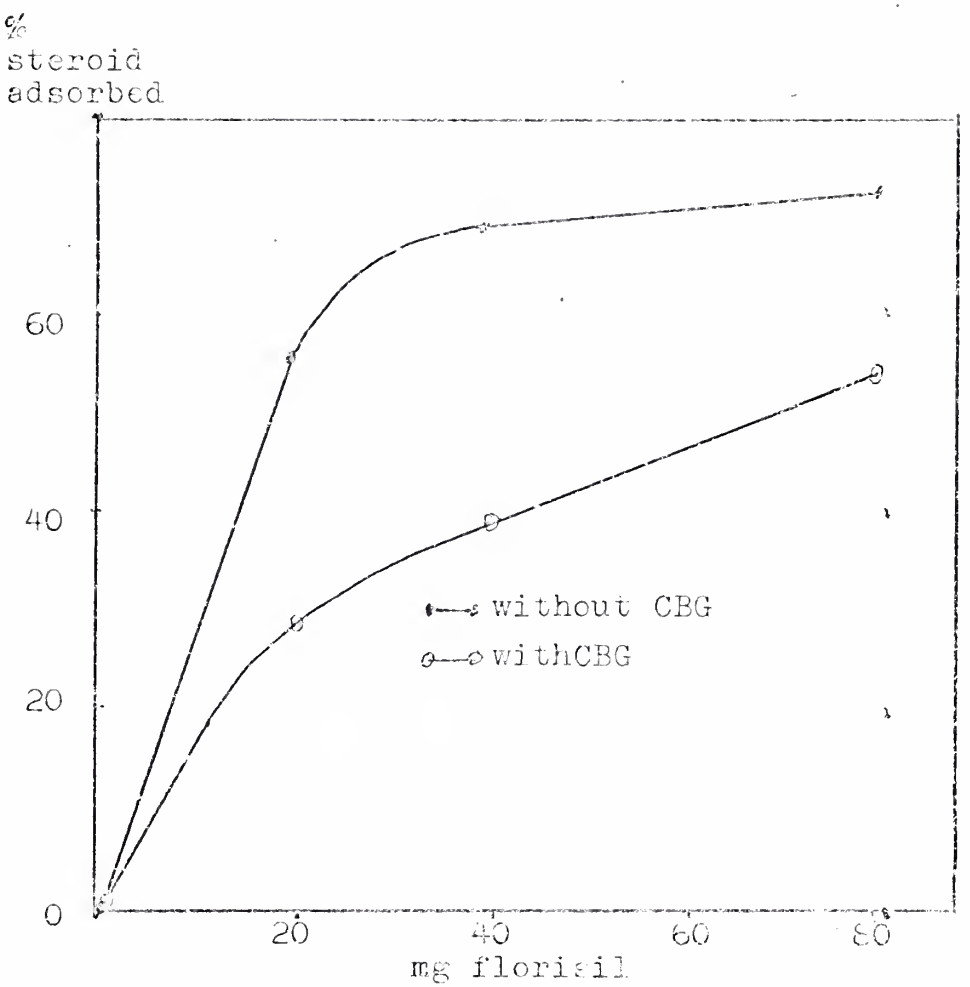


Plate VII-- Regions of non--linearity in standard curves
Means and extreme values plotted at each coordinate

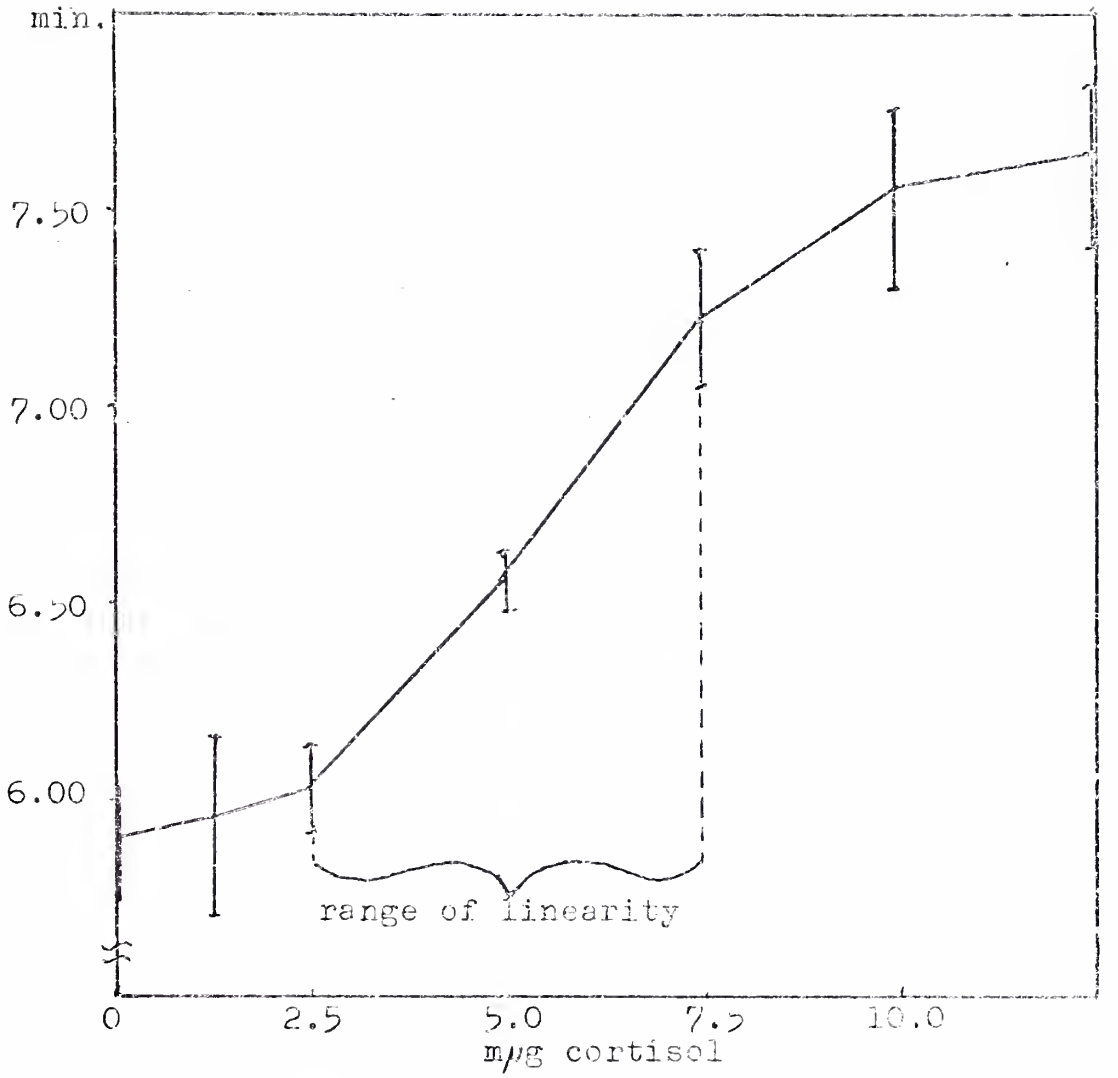
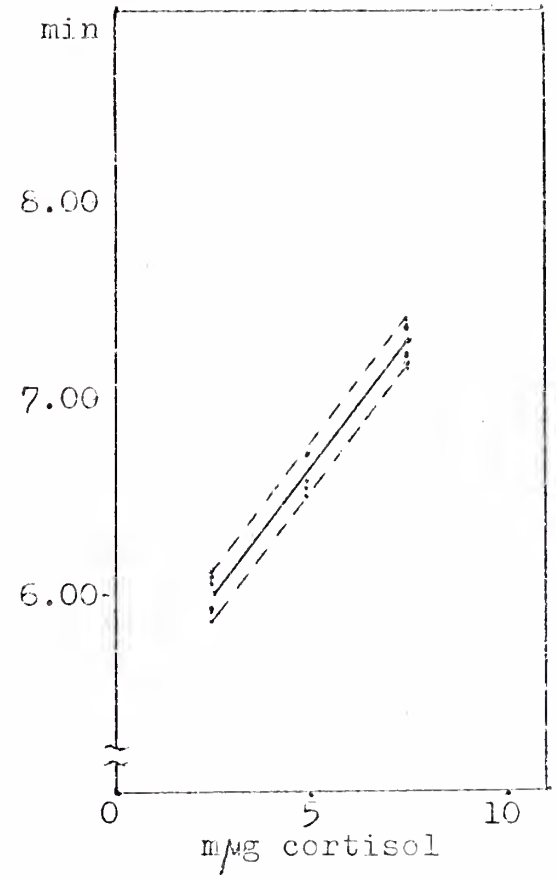


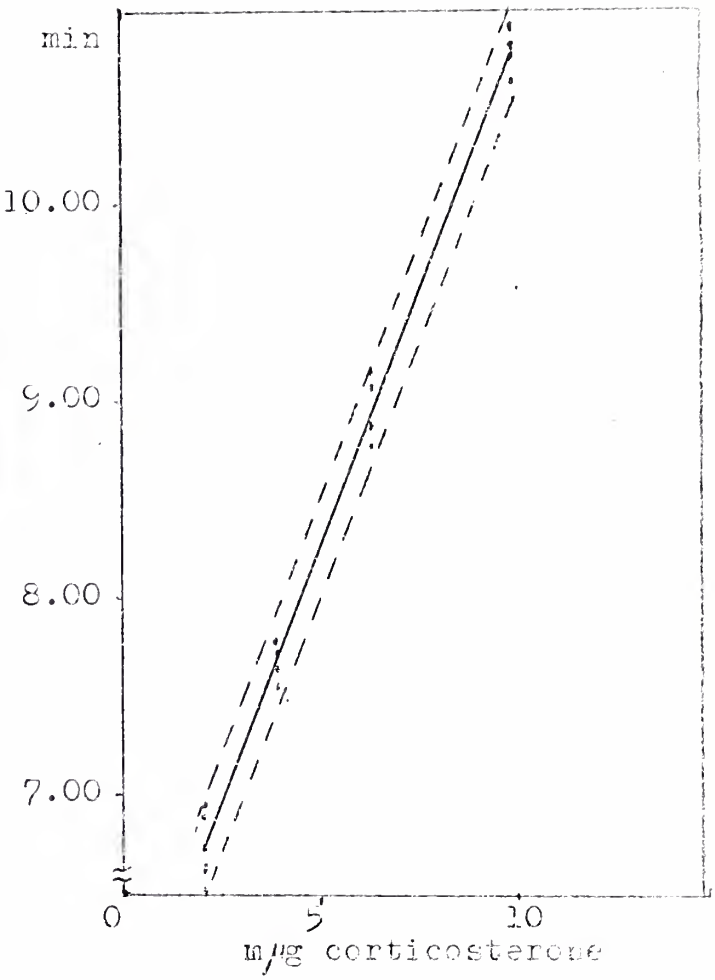
Plate VIII- Representative values for standard curves

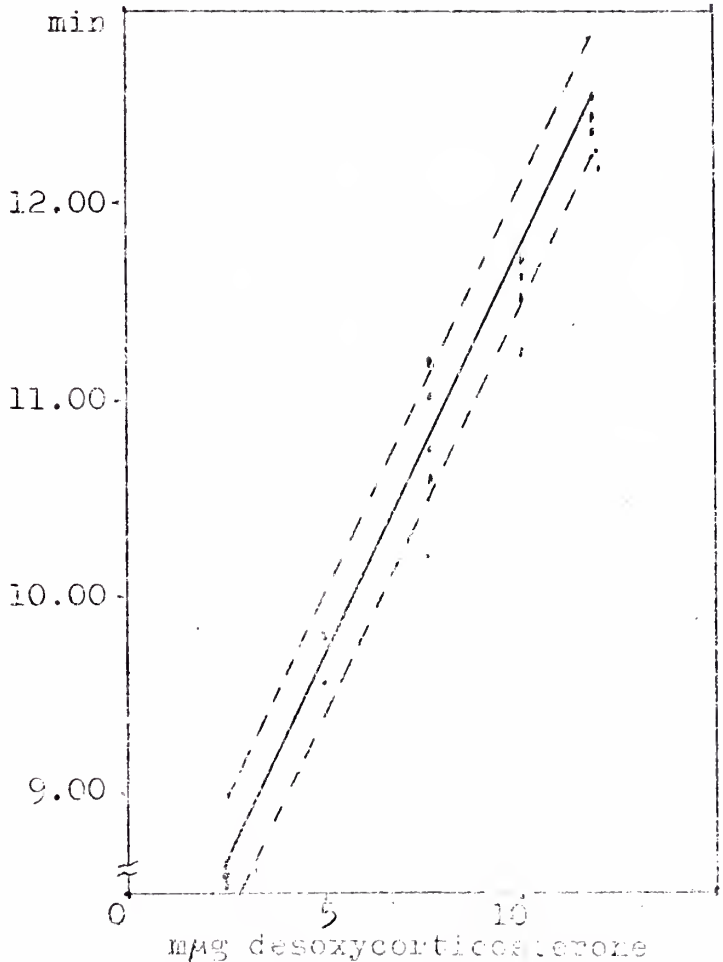
steroid	range of linearity m/g	slope min/m/g	standard deviation min
cortisol	2.5-7.5	.260	.122
corticosterone	2-10	.519	.175
desoxy-cortisol	2-10	.332	.262
desoxy-corticosterone	2.5-12.5	.372	.246

Plate IX — Representative standard curves

——line of best fit - - - ± 1 standard deviation







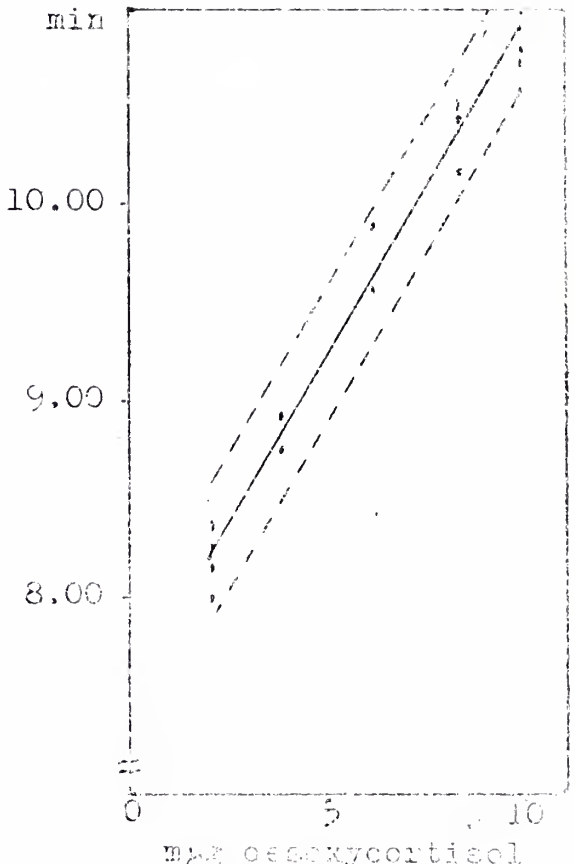


Plate X -- Concentrations of steroids in fetal tissues

means ± 95% confidence limits

Tissue	days of gestation	cortico-sterone mμg/gm	desoxy-cortico-sterone mμg/gm	cortisol mμg/gm	desoxy-cortisol mμg/gm
placentae	18	3.04±6.68	3.82±2.46	2.78±10.1	-
	19	3.09±2.73	3.10±1.77	2.29±7.97	-
	20	6.87±6.58	-	4.10±4.40	-
	21	1.83±2.51	-	25.1±4.58	-
	22*	-	-	-	-
fetal bodies	18	2.54±2.96	1.34±1.36	30.0±11.1	-
	19	2.92±3.87	1.36±0.79	1.20±3.30	-
	20	1.54±1.53	0.92±0.45	19.5±5.50	-
	21	2.76±0.59	0.55±0.33	11.4±5.27	-
	22*	-	-	-	-
fetal adrenals	18	1886±533	1572±470	2183±1057	-
	19	7505±1469	-	287±173	-
	20	-**	-**	-**	-**
	21	1051±692	-	-	-
	22*	745±533	-	-	-

*newborn

**inadequate tissue sample

Plates XI & XII -- Concentrations of steroids in fetal tissues

Graphic form

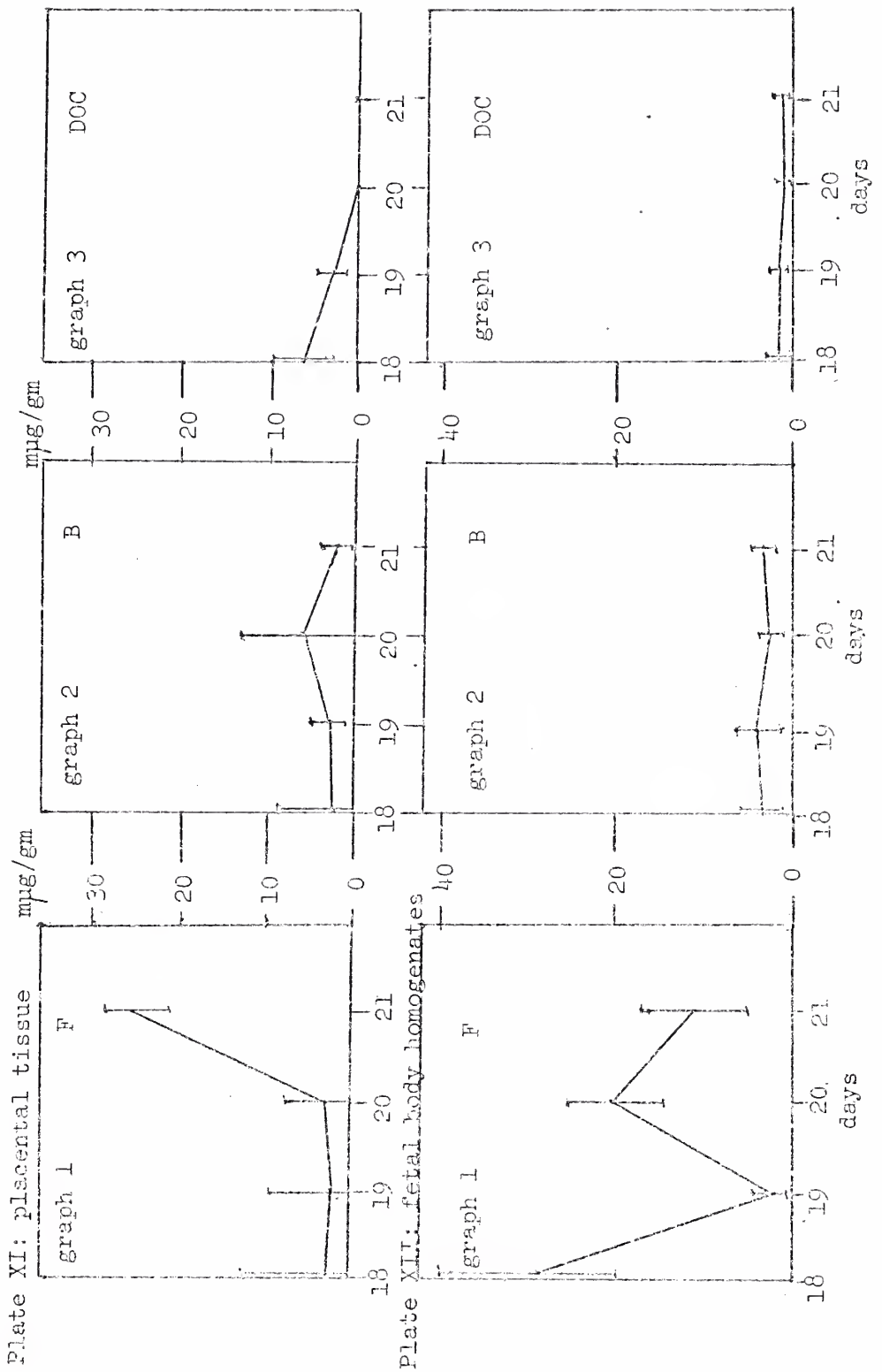


Plate XIII -- Concentrations of steroids in fetal adrenal tissue

Graphic form

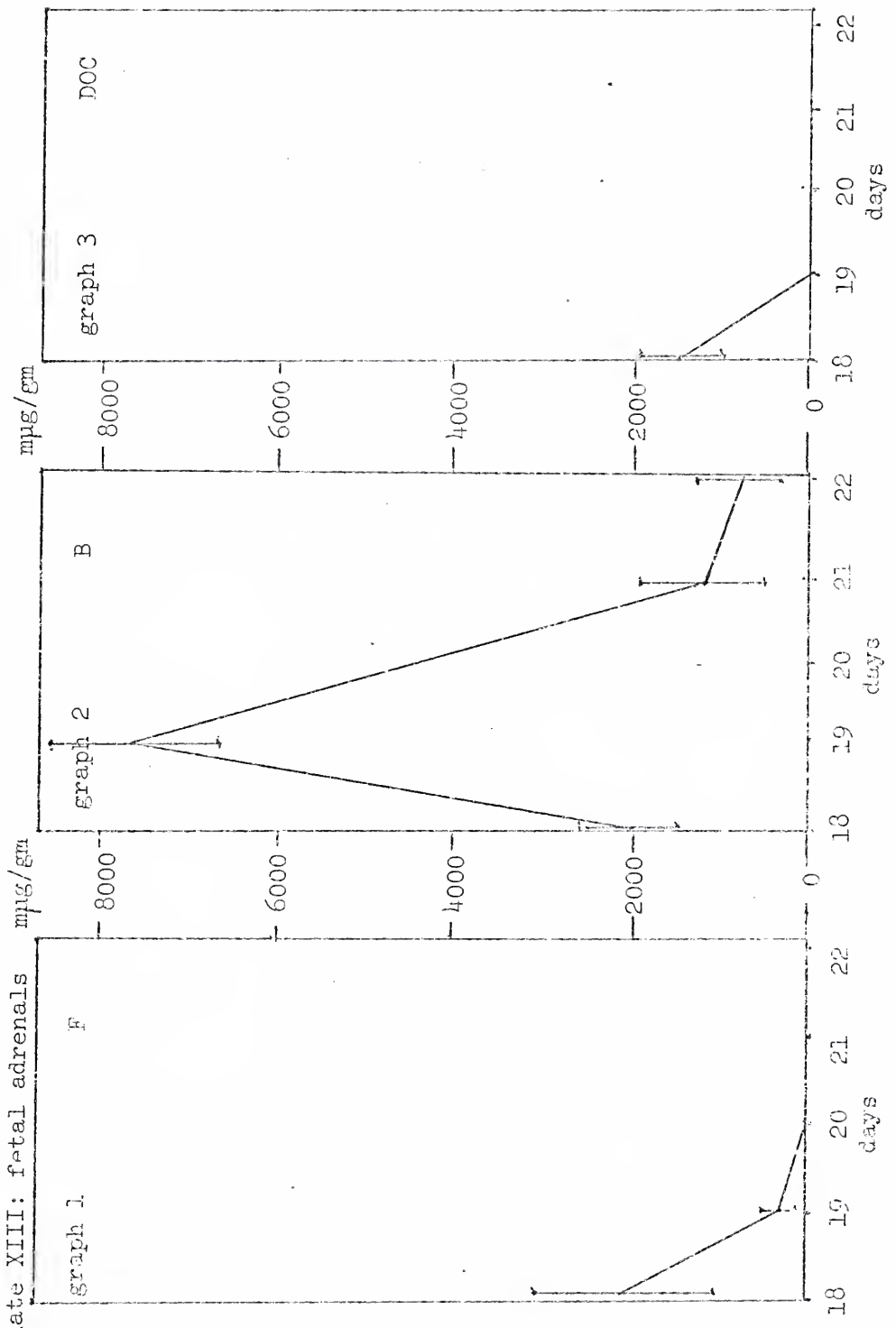
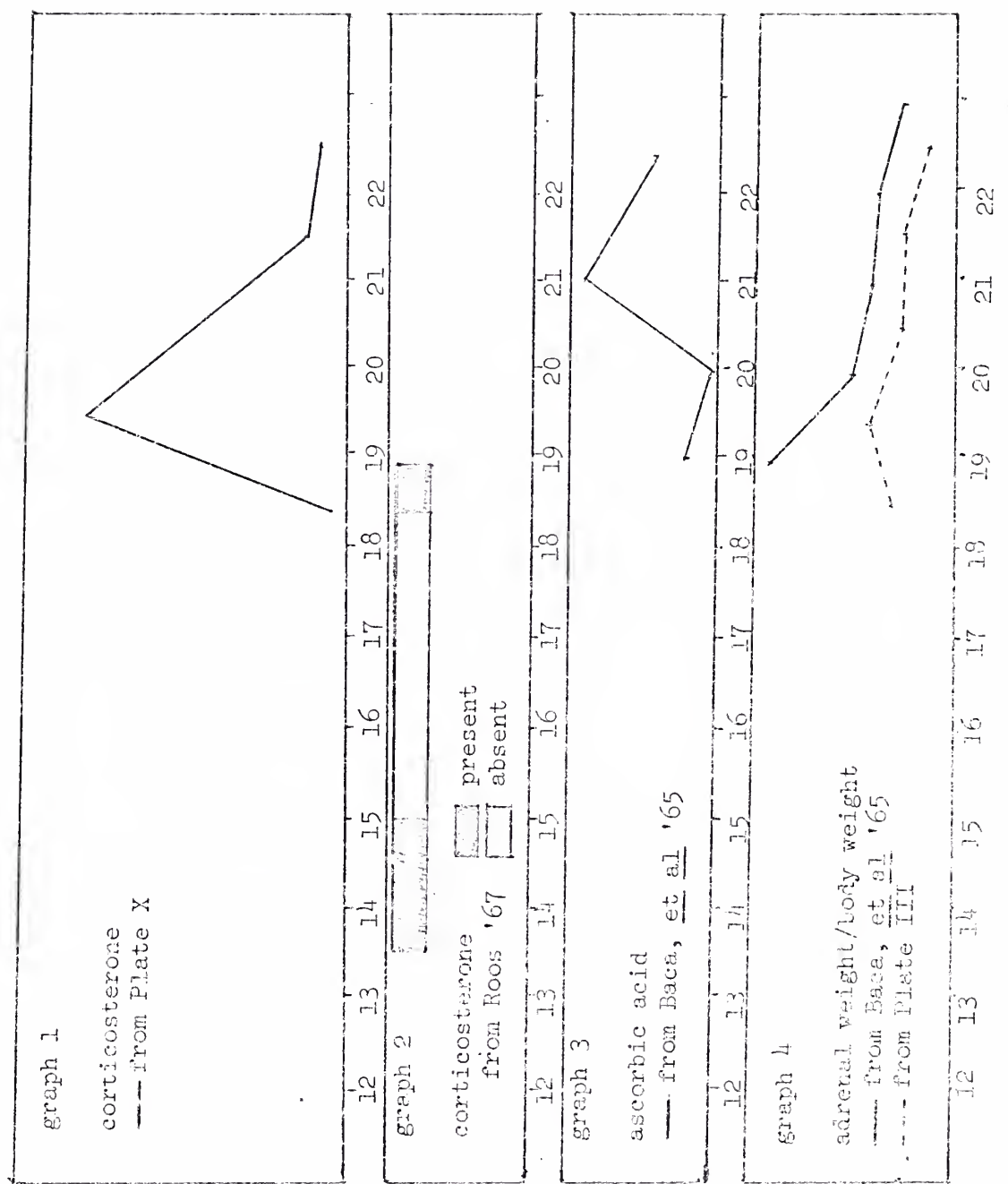


Plate XIV: biochemical parameters of steroidogenesis



BIBLIOGRAPHY

1. Alov, I.A., On the Relation Between Mitosis and the Functional Activity of Cells of the Adrenal Cortex, Biull. Eksp. Biol. Med. 54:87-91, 1962.

2. Angelico, R., Cavina, G., D'Antona, A., Giocoli, G., Fractionation and Determination of the Lipid and Steroid Constituents of the Adrenal Glands of Rats by Means of Thin-layer Chromatography, J. Chromatog. 18:57-68, 1965.

3. Baca, Z.V., Chiodi, H., Developmental Changes in Size and Ascorbic Acid Content of the Adrenals of White Rats. Endocrinology 76:1208-1212, 1965.

4. Bentley, P.J., The Adrenal Cortex and Renal Sodium and Potassium excretion in the Newborn Rat, J. Endocr. 26:361-365, 1963.

5. Braunsberg, H., James, V.H.T., The Determination of Adrenocortical Steroids in Blood, J. Endocr. 21:327-340, 1960.

6. Brownlee, K.A., Statistical Theory and Methodology, John Wiley & Sons, Inc., New York, N.Y., 1960.

7. Bush, I.E., Species Differences in Adrenocortical Secretion, J. Endocr. 9:95-100, 1953.

8. Chieffi, C., Organogenesis, de Haan, R.L., Ursprung, H., (eds), Holt, Rinehart & Winston, New York, N.Y., 1965.

9. Cohen, A., Variations de la teneur en lipides de la corticosurrenale foetale du Rat, an cours du developpment, C. R. Soc. Biol. (Paris) 148:321-324, 1954.

10. Cohen, A., Repartition des lipides dan le cortex surrenal d'embryous de Rats Decapites, Arch. Anat. micr. Morph. exp. 44:265-276, 1955.

11. Cohen, A., Poids des surrenales du foetus de Rat decapite injecte d'hydrocortisone on de cortico-stimuline a divers stades du developpment, C. R. Soc. Biol. (Paris) 154:1396-1400, 1960.

12. Contopoulos, A.N., Simpson, M.E., Presence of Trophic Hormones in Foetal Rat Hypophysis, Fed. Proc. 16: 24, 1957.

13. Dixon, P.F., Booth, M., Butler, J., "The Corticosteroids," Hormones in Blood, Vol. 2, Gray.C.H., Bacharach, A.L., (eds), Academic Press, New York, N.Y., 1967.

14. Dobrokhotov, V.N., Nikanorova, R.I., Diurnal Periodicity of Mitotic Division of Adrenal Cells of Albino Rats, Bull. Exper. Biol Med. 54:1027-1032, 1963.

15. Domm, L.V., Leroy, P., A Method for Hypophysectomy of the Rat Fetus by Decapitation, Anat. Rec. 109:395-396, 1951.

16. Givner, M.L., Rochfort, J.G., An Improved Assay of Corticosterone in Rat Serum and Adrenal Tissue, Steroids 6:485-489, 1965.

17. Holzbauer, M., A Comparison of the Concentrations of Steroids in the Adrenal Gland with the Rates of Their Secretion, Naunyn. Schmiedeberg Arch. Pharm. Exp. Path. 259:173, 1968.

18. Jailer, J.W., The Maturation of the Pituitary-Adrenal axis in the Newborn Rat, Endocrinology, 46:420-425, 1950.

19. Jailer, J.W., Wong, A.S.H., Engle, E.T., Pituitary-Adrenal Relationship in Full Term and in Premature Infants, as Evidenced by Eosinophil Response, J. Clin. Endocrinol. 11:186-192, 1951.

20. Josimovich, J.B., Ladman, A.J., Deane, H.W., A Histophysiological Study of the Developing Adrenal Cortex of the Rat during Fetal and Early Postnatal Stages, Endocrinology 54:627-639, 1954.

21. Jost, A., Influence de la decapatation sur le developpment du tractus genital et des surrenales de l'embrou de Lapin, C. R. Soc. Biol. 142:273-275, 1948.

22. Jost, A., Hormonal Factors in the Development of the Foetus, Cold Spring Harb. Symp. Quant. Biol. 19: 167-180. 1954.

23. Jost, A., The Secretory Activities of Fetal Endocrine Glands and their Effect upon Target Organs, Josiah Macy Foundation 3rd Conf. on Gestation, Princeton, 1956.

24. Jost, A., The Age Factor in some Prenatal Endocrine Events, Cibs Found. Colloq. Aging 2:18-30, 1956.

25. Jost, A., Jacquot, R., Cohen, A., "The Pituitary Control of the Foetal Adrenal Cortex," The Human Adrenal Cortex, Currie, A.F., (ed), The Williams & Wilkins Co., Baltimore, Md., 569-579, 1962.

26. Kitchell, R.O., Wells, L.J., Functioning of the Hypophysis and Adrenals in Fetal Rats, Anat. Rec. 112: 561-591, 1952.

27. Kitchell, R.L., Wells, L.J., Reciprocal Relation between the Hypophysis and Adrenals in Fetal Rats: Effects of Unilateral Adrenalectomy and of Implanted Cortisone, DOCA, and Sex Hormones, Endocrinology 50:83, 1952.

28. Levine, S., Lewis, G.W., Critical Period for Effects of Infantile Experience on Maturation of Stress Response, Science 129:42-43, 1959.

29. Levine, S., Mullins, R.F., Jr., Hormonal Influences on Brain Organization in Infant Rats, Science 152: 1585-1592, 1966.

30. Milkovic, K., Milkovic, S., Functioning of the Pituitary-Adrenocortical Axis in Rats at and after Birth, Endocrinology 73:535-539, 1963.

31. Moog, F., "The Development of Function in the Adrenal Cortex," Comparative Endocrinology, Gorbman, A., (ed), Wiley, New York, N.Y., 624-638, 1959.

32. Muhlemann, H.R., Marthaler, T.M., Loustalot, P., Daily Variations in Mitotic Activity of Adrenal Cortex, Thyroid and Oral Epithelium of the Rat, Proc. Soc. Exp. Biol. Med. 90:467-468, 1955.

33. Muhlemann, G.R., Marthaler, T.M., Rateitschak, K.H., Acta. Anat. Basel 28: 331, 1956.

34. Murphy, B.E.P., Some Studies on the Protein-Binding of Steroids and their application to the Routine Micro and Ultramicro Measurement of Various Steroids in Body Fluids by Competitive Protein-Binding Radioassay, J. Clin. Endocr. 27:973-991, 1967.

35. Murphy, B.E.P., "The Determination of Plasma Levels of Corticoids and their Diagnostic significance," Introduction to Clinical Endocrinology, Bajusz, E., (ed), S. Karger, Basel/New York, N.Y., 1967.

36. Noumura, J., Development of the Hypophyseal-thyroidal System in the Rat Embryo and its Relation to the Maternal System, Japan J. Zool. 12:301-318, 1959.

37. Palkovitz, M., Mitro, A., Morphological Changes in Hypothalamo-pituitary-adrenal System during Early Postnatal Period in Rats, Gen. Comp. Endocr. 10: 253-262, 1968.

38. Picon, R.O., Action de l'hypophyse et des surrenales maternelles sur le poids des surrenales du foetus de Rat, C. R. Soc. Biol. (Paris) 152:1469-1472, 1958.

39. Rapkin, E., Int. J. Appl. Radiat. 15:69, 1964.

40. Roos, T.R., Steroid Synthesis in Embryonic and Fetal Rat Adrenal Tissue, Endocrinology 81:716-728, 1967.

41. Russell, J.A., "The Adrenals," Physiology and Biophysics, Ruch, T.C., Patton, H.D., (eds), W.B. Saunders Co., Philadephia, Pa., 1965.

42. Sakatskaya, T.S., Biosynthesis of Corticosteroids by Rat Embryonic Adrenals, Prob. Endokr. Gormonoten 10:67-70, 1964.

43. Skebelskaya, Y., The Effect of Corticosteroid Concentration in the Blood of Gravid Rats on the Adrenocorticotropin Function of the Hypophysis of the Fetus, Gen. Comp. Endocr. 10:263-268, 1968.

44. Telegdy, G., Endroczi, E., Estimation of Adrenal Function in vivo and in vitro: Comparison of the Two Methods of Assay in the Rat treated with ACTH, Acta. Physiol. Humg. 28:133-137, 1965.

45. Van Dorpe, W.V., Deane, H.W., A Morphological and Cytochemical Study of the Postnatal Development of the Rat's Adrenal Cortex, Anat. Rec. 107:265-281, 1950.

46. Villee, D.B., Engel, L.L., Loring, J.M., Villee, C.A., Steroid Hydroxylation in Human Fetal Adrenals: Formation of 16 a-hydroxyprogesterone, 17 hydroxyprogesterone and Deoxycorticosterone, Endocrinology 69:354-362, 1961.

47. Wells, L.J., Progress Studies Designed to Determine Whether the Fetal Hypophysis produces Hormones that Influence Development, Anat. Rec. 97:409, 1947

48. Wells, L.J., Some Experimental Evidence of Production of Adrenotrophin by the Fetal Hypophysis, Proc. Soc. Exp. Biol. & Med. 68:487-488, 1948.

49. Wells, L.J., Microscopical Study of Acrenals of Fetuses Deprived of the Hypophysis and Given Adrenocorticotropin, Anat. Rec. 103:563-564, 1949.

50. Witschi, E., Biological Handbook: Growth, Altman, P.L., Dittmer, D.S., (eds), W.B. Saunders Co., Philadelphia, Pa., p.308, 1962.

51. Yamane, T., Statistics: An Introductory Analysis, Harper & Row, New York, N.Y., 1967.

52. Zarrow, M.X., Haltmeyer, G.C., Denenberg V.H., Thatcher, J., Response of Infantile Rat to Stress, Endocrinology 79:631-634, 1966.

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